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Permalink

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Journal

Journal of veterinary internal medicine, 28(1)

ISSN

0891-6640

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Publication Date

2014

DOI

10.1111/jvim.12256

Peer reviewed

Identification of PDE5A:E90K: A Polymorphism in the Canine Phosphodiesterase 5A Gene Affecting Basal cGMP Concentrations of Healthy Dogs

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Background: Cyclic guanosine monophosphate (cGMP)-specific phosphodiesterase (PDE5A) is the target of phosphodiesterase inhibitors such as sildenafil. Polymorphisms in the PDE5A gene that may predict response to therapy with sildenafil and nitric oxide, be linked to disease progression, and aid in risk assessment have been identified in human beings. Identification of polymorphisms in PDE5A could affect the physiologic actions of PDE5A and the effects of phosphodiesterase type 5 inhibitor drugs.

Hypothesis/Objective: Functional polymorphisms exist in the canine PDE5A gene. Specific objectives were to identify PDE5A polymorphisms and evaluate their functional relevance.

Animals: Seventy healthy dogs.

Methods: The exonic, splice-site, 3' and 5' untranslated regions of the canine PDE5A gene were sequenced in 15 dogs and aligned with the canine reference sequence. Identified polymorphisms were evaluated in 55 additional, healthy, unrelated dogs of 20 breeds. Plasma was collected from 51 of these dogs and cGMP was measured. An unpaired *t*-test and one-way ANOVA with Dunnett's test of multiple comparisons were used to evaluate the effect of genotype on cGMP.

Results: A common exonic polymorphism was identified that changed glutamic acid to lysine and resulted in significantly lower cGMP concentrations in the group with polymorphism versus the wild type group ($P = .014$). Additionally, 6 linked single nucleotide polymorphisms in the 3' untranslated region were identified that did not alter cGMP concentrations.

Conclusions and Clinical Importance: A polymorphism exists in the canine PDE5A gene that is associated with variable circulating cGMP concentrations in healthy dogs and warrants investigation in diseases such as pulmonary hypertension.

Key words: Individualized medicine; Pharmacogenomics; Pulmonary hypertension; Sildenafil citrate; Single nucleotide polymorphisms.

Pharmacogenetics (genetic differences that affect an individual's response to a drug) has become an important discipline in the management of many human disease processes and is driving individualized medicine.¹ Despite wide variations in clinical response to many pharmacologic agents, limited reports of genetic polymorphisms and pharmacogenomic effects exist in veterinary medicine.^{2–4}

Examples of the importance of a pharmacogenetic relationship in human beings are polymorphisms in the phosphodiesterase 5A (PDE5A) gene that may predict response to therapy with sildenafil and nitric oxide, be linked to disease progression, and aid in risk assessment in childhood IgA nephropathy, pulmonary hypertension (PH), systemic hypertension, and erectile dysfunction.^{5–7} The PDE5A gene encodes a cyclic guanosine monophosphate (cGMP)-specific

Abbreviations:

cGMP	cyclic guanosine monophosphate
CKCS	Cavalier King Charles Spaniel
PCR	polymerase chain reaction
PDE5A	cyclic guanosine monophosphate specific phosphodiesterase
PDE5-I	phosphodiesterase type 5 inhibitors
PH	pulmonary hypertension
SNP	single nucleotide polymorphism
UTR	untranslated region

phosphodiesterase. It is a key physiologic regulator of cGMP and pharmacologic target of vasodilatory drugs (such as sildenafil citrate) used commonly to treat PH and erectile dysfunction.⁸ The coding sequence, functional regions, and transcript variants of PDE5A are well described in human beings.^{9,10} Additionally, the genetic sequence of phosphodiesterase genes has been demonstrated to be responsible for the selective action of some phosphodiesterase inhibiting agents. When gene segments are altered or removed, phosphodiesterase inhibiting agents may no longer be able to exert their actions.¹¹

Similar to human beings, PDE5A and drugs that inhibit its action represent a key concept in the pathophysiology and treatment of PH in veterinary medicine. Pulmonary hypertension is a cause of respiratory distress and collapse in dogs and defined as systolic pulmonary arterial pressure >25 mmHg.¹² It is a pathologic response to several pulmonary parenchymal diseases and parasitic insults, and may develop secondary to increased left atrial pressure, pulmonary blood flow,

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Submitted June 5, 2013; Revised September 1, 2013; Accepted October 22, 2013.

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10.1111/jvim.12256

or increased pulmonary vascular resistance.¹² In studies of the most common acquired canine heart disease (mitral valve degeneration), 14–31% of cases have concurrent PH.^{13,14} Additionally, up to 20% of dogs with PH are reported to suffer from syncopal events.¹² Although it occurs secondary to multiple inciting causes and several classification schemes have been described, the overall prognosis and response to therapy are poor in dogs with the severe form of the disease.¹⁵ In one study, median survival time was only 91 days.¹⁶ In many cases of severe PH, dogs are euthanized because of failure to respond to therapy and progressive respiratory distress.^{15–17} Phosphodiesterase type V inhibitors (PDE5-I) are commonly prescribed for PH because they increase pulmonary vascular concentrations of cGMP, which in turn promote endogenous nitric oxide concentrations and resultant vasodilatation.^{15–17}

We propose genetic polymorphisms as a possible explanation for the variable response to treatment of PH in dogs and this study represents the first investigation of PDE5A gene variation in dogs.

We hypothesized that functional polymorphisms exist in the canine PDE5A gene. Our specific aims included evaluation of canine PDE5A polymorphisms, prediction of their structural consequence by computer modeling, and evaluation of their functional relevance in a healthy canine population by measurement of cGMP.

Materials and Methods

DNA samples from 15 unrelated, apparently healthy dogs of 3 breeds (5 Golden Retrievers, 5 Norwich Terriers, and 5 Cavalier King Charles Spaniels [CKCS]) were evaluated. These breeds were chosen from the university DNA library specifically to determine if there was any appreciable difference between genotypes of large breed dogs (golden retrievers) versus small breed dogs likely to be affected by mitral valve degeneration and consequently PH (CKCS, Norwich Terriers). Genomic DNA samples were prepared from whole blood samples as previously described.¹⁸ Briefly, cells were osmotically lysed in 2× sucrose-Triton (pH 7.6) and Tris-NH₄Cl (pH 7.2) buffer, and nuclei were pelleted by centrifugation at 380 × g for 20 min at 25°C. Pellets were resuspended in saline-EDTA (pH 8.0) with 20% sodium dodecyl sulfate (SDS) and 600 mAU/mL proteinase K, and incubated overnight at 56°C. The samples were subjected to 2 successive phenol:chloroform:isoamyl (25:24:1, pH 8) and 1 chloroform extraction. Finally, DNA was precipitated (in 95–100% ethanol and 2 M NaCl) and resuspended in 100–200 µL of Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8). Genomic DNA was quantified and assessed for quality and purity based upon spectrophotometry.^a

Polymerase chain reaction (PCR) amplification primers were designed for all exons, splice site regions, 3' untranslated (1,200 bp), and 5' untranslated regions (500 bp) of the canine PDE5A gene (ENSCAFG00000012472) using Primer 3 software (<http://frodo.wi.mit.edu/>) and the canine nucleotide sequences from the Ensembl genomic database (<http://www.ensembl.org/index.html>; Table 1). Primers were designed to include the gene promoter region and cover at least 500 upstream nucleotides before the first reported exon. PCR was carried out using a 25 µL cocktail of molecular grade water, 10× KCL-containing

Table 1. PDE5A primer design. The exon or region in PDE5A is displayed with its corresponding forward and reverse primer utilized for PCR and sequencing.

Exon or Region	Forward Primer Sequence	Reverse Primer Sequence
5' UTR	cgagggtttgtggatgtgtc	gcaacatagcgcagcacagaa
1	gcgggtgggtcagtgag	ggaactacctcttttggtgccaa
2	gcagcttcagagagatagca	tctctctcaccacctac
3	tcagctcttgaacaggtcagtc	aaaagaaaatgtttcaaatgacc
4	tttcagccaatgaaactacca	tcgctatcaacaatttcagca
5	ttggctgttgcttacctgtttt	gctaaaatgttaactggagttaatac
6	taatgagctctaaattttcctgtct	ggctaacaatgaagttaaaagcagt
7	ccacaagtattggtgtttgtg	ttcagtcgtcgtgttcaagg
8	ctctgttttgcctatgtttt	aagtcgagatctgatgggaaa
9	agcacctaattggacaaatcaa	tcaaatttacaactgaaggaaaaa
10	tttgctcaatttcttgattgtt	cagagatcgagagcgtctctg
11	cattcgtagccctctcttg	gatgcctcagttagtcactttt
12	tttgtaatcactgctgtt	tccaaggacatgattgtattcc
13	tgtacgtgttagcaagcattatt	attccaatcattgttagtgcaa
14	cccatgtgaaaaacactcagaa	ctggggaaaacctgccttac
15	ttgtgcagtagccgtttt	cccttttaaaattccacaacca
16	gcttctccaagtggagtgct	tgcacaacacttccaagacct
17	gagcctggggactcatcata	tctgacagcctcgaagatca
18	ttggctctttttgctcta	gactcaatccagggtctcca
19	ttttactacgtggttgaaagc	ttctaccaccaaggtctca
20	gcattgttttgagccaact	tttagcaagtcgtgttttca
21	gggagagggctatttctctg	caaaaacctacctcagtgcaa
3' UTR	ccaccactcttagcacaca	cacctcaagtcaatgtccca
(section 1)		
3' UTR	tctctgagagtcctgttttga	caatgttttagcattttctatatgtgc
(section 2)		
3' UTR	cacggaagtgtgggtgtgta	aaaagttctttgagggtgctg
(section 3)		

Taq buffer, 1 mM MgCl₂, 0.5 units/µL of reaction volume Taq DNA Polymerase,^b 0.4 mM dNTPs, 0.4 µM PCR amplification primers, and 100–200 µg DNA. The PCR protocol included 5 min at 95°C, 40 cycles of 94°C for 30 s, 57°C for 30 s, 72°C for 30 s, and a final extension phase at 72°C for 7 min. The annealing temperature was optimized for respective primers (50–66°C). A water control (blank) with no DNA added was run with each PCR reaction to confirm absence of DNA contamination. Successful PCR amplification was verified by loading 5 µL of each PCR product and blank with 1 µL of agarose loading dye on a 1.5% agarose gel and performing electrophoresis. The amplification band was visualized by ultraviolet light to confirm presence of the appropriately sized product for each corresponding PCR primer set and the absence of any bands in the water controls. Products were sequenced with both forward and reverse primers and analyzed on a sequencer.^c

Nucleotide sequences were evaluated visually for sequence quality and aligned using software^d to evaluate for DNA variants among the individual animals. Any variants identified were evaluated to determine if they changed the amino acid (nonsynonymous polymorphism) produced from the reference protein sequence from the Ensembl Browser (<http://www.ensembl.org/index.html>) and were in the same location as any known human variants obtained from the Pharmacogenomics Knowledgebase (<http://www.pharmgkb.org/>). If amino acid changes were observed, their relevance was evaluated with the PolyPhen-2 program (<http://genetics.bwh.harvard.edu/pph2/>) to predict possible functional significance and determine the conservation of this protein region across species.¹⁹ Additionally, functional relevance was predicted by use of the Mutation Taster program

(www.mutationtaster.org), which reports likelihood of a mutation to cause disease, predicts functional consequences and scores the prediction for accuracy using a Bayes classifier.^{20,21}

Any identified polymorphism that was predicted to be functionally relevant then was evaluated in a larger cohort of apparently healthy dogs, and the genotypes were recorded. This sample group included an additional 55 unrelated dogs of 19 different breeds (4 additional Golden Retrievers, 1 additional CKCS, 7 Labrador Retrievers, 5 American Staffordshire Terriers, 4 Miniature Dachshunds, 3 Jack Russell Terriers, 2 Greyhounds, 2 German Shepherds, 2 German Shorthair Pointers, 1 Standard Poodle, 1 Australian Shepherd, 1 English Mastiff, 1 Boxer, 1 Basenji, 1 English Setter, 1 Border Collie, 1 German Wirehair Pointer, 1 Chihuahua, and 16 mixed breed dogs). Additionally, a whole-blood sample was collected in a citrated tube, and plasma was immediately obtained by centrifugation and frozen at -80°C in 51 of these dogs. A commercially available multi-species cGMP EIA measurement kit^e was validated for use in this dog group by confirming intra-assay precision (coefficient of variation $<10\%$), dilution linearity, and spike recovery in pooled canine plasma. cGMP was measured in plasma for each of the 51 samples in duplicate by spectrophotometry and standard curve methodology as previously described.²² Samples with $>10\%$ coefficient of variation were repeated. cGMP concentrations were separated by genotype as either wild type (matching the canine reference sequence) or polymorphic (either positive heterozygous or positive homozygous for the polymorphism). The median, 25th and 75th percentiles are reported for each genotype category tested. Results were evaluated statistically with an unpaired *t*-test and significance was set at an alpha of <0.05 using commercially available software.^f A one-way ANOVA with Dunnett's multiple comparison posttest to compare genotype groups versus the control group (wild type versus heterozygous; wild type versus homozygous) also was performed.

Results

A series of 5 linked single nucleotide polymorphisms were identified in the 3' untranslated region as follows: PDE5A:g.129467A>G; g.129281T>C; g.129858C>A; g.130270T>C; g.130311G>C; g.130515A>C. Although SNPs within the 3' UTR of human beings previously have been reported and linked to progression of some diseases, none of the identified canine SNPs in the 3' UTR region previously have been identified in human beings.⁶ This 3' UTR SNP set of polymorphisms was identified in a 44/70 dogs (11 homozygous, 33 heterozygous, 26 wild type). Polyphen2 and Mutation Taster predicted these SNPs to be benign and no changes to splice sites or protein features were expected.

A single genomic variant was identified that changes a guanine to adenine in the nucleotide sequence. This change represents an exonic polymorphism and is predicted to change the protein's amino acid sequence in exon 2 from glutamic acid (E) to lysine (K) in the polymorphic dogs (PDE5A:E90K). This change is not reported in human beings. The reference amino acid, glutamic acid, is acidic in nature, where as lysine is basic. This change thus is predicted to alter the acid-base status of the amino acid sequence within this region of the protein from acidic to basic and was predicted to be possibly damaging by Polyphen-2 with a HumDiv score of 0.763 (0–0.45 is benign; 0.45–0.95 possibly damaging, 0.95–1.0 probably damaging).¹⁹

The PDE5A:E90K polymorphism also changes the amino acid within a highly conserved region of the PDE5A gene across species (Table 2). Mutation Taster evaluation predicted this alteration to be damaging with an excellent probability score (0.9635/1.0).^{20,21} Additionally, Mutation Taster predictions find this amino acid substitution to result in splice site changes and possibly to alter the protein features.^{20,21} The PDE5A:E90K polymorphism was identified in 61/70 dogs analyzed (21 homozygous, 40 heterozygous, 9 wild type). Known functional domains are described for phosphodiesterase genes and PDE5A in human beings and dogs. The PDE5A:E90K change is upstream, adjacent to the GAF1 domain in the PDE5A gene as reported with direct transcript evidence in the UniprotKB database (www.uniprot.org). Previously reported exonic SNPs in human beings are reported within the GAF1 domain, but are predicted to be benign in comparison with those reported here.⁶

Plasma concentrations of cGMP in 51 healthy, unrelated dogs (18 wild type, 18 heterozygous and 15 homozygous) demonstrated a significant difference between PDE5A:E90K wild type and pooled polymorphic dogs (wild type versus combined heterozygous and homozygous, $P = .014$, Fig 1), but no statistical difference between age, sex or genotype of the 3' UTR SNP set. The median (25th and 75th percentiles) of cGMP concentration of polymorphic dogs was 1.52 (1.22; 2.07) pmoles/mL, compared to wild type dogs measured at 1.87 (1.60; 2.43) pmol/mL. When divided into genotype classes, the heterozygous variant median cGMP measured 1.57 (1.31; 1.88) pmol/mL, whereas the homozygous variant median was 1.52 (0.75; 2.25). One-way ANOVA also identified a significant difference among PDE5A:E90K genotype groups ($P = .045$) with Dunnett's test of multiple comparisons identifying wild type versus homozygous comparison as significant.

Table 2. PDE5A:E90K conservation evaluation. The amino acid sequence surrounding the PDE5A:E90K polymorphism is displayed in various species. The glutamic acid at position 90 (E) is highly conserved among highly varied species and seen to be substituted by a lysine (K) in the polymorphic dog sequence only.

Species	Amino Acid Sequence
Rat	T V S F L A D S E K K E Q M P L T P P R F D N
Platypus	T V S F L A D S E K K E Q M P L T P P R F D N
Opossum	T V S F L A D S E K K E Q M P L T P P R F D N
Chameleon	T V S F L A D S E K K E Q M P L T P P R F D N
Turkey	T V S F L A D S E K K E Q M P L T P P R F D N
Chicken	T V S F L A D S E K K E Q M P L T P P R F D N
Pufferfish	T V S F L A D S E K K E Q M P L T P P R F D N
Frog	T V S F L A D S E K K E Q M P L T P P R F D N
Human	T V S F L S D S E K K E Q M P L T P P R F D N
Cat	T V S F L S D S E K K E Q M P L T P P R F D N
Horse	T V S F L S D S E K K E Q M P L T P P K F D N
Elephant	T V S F L S D S E K K E Q M P L T P P R F D N
Dog	T V S F L A D S E K K E Q M P L T P P R F D N
Mutant Dog	T V S F L S D S E K K K Q M P L T P P R F D

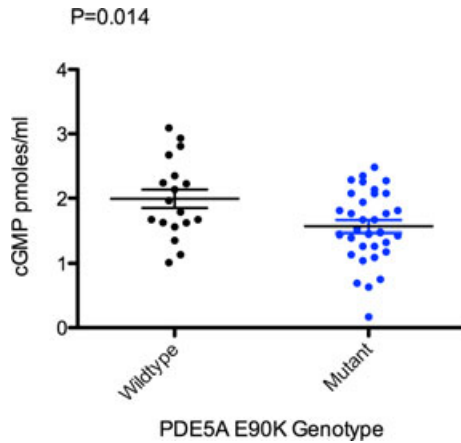


Fig 1. Basal cyclic guanosine monophosphate (cGMP) concentrations of healthy dogs are charted by either wild type or polymorphic (heterozygous and homozygous) genotype. An unpaired *t*-test determined these groups to be statistically different. The *P*-value is shown.

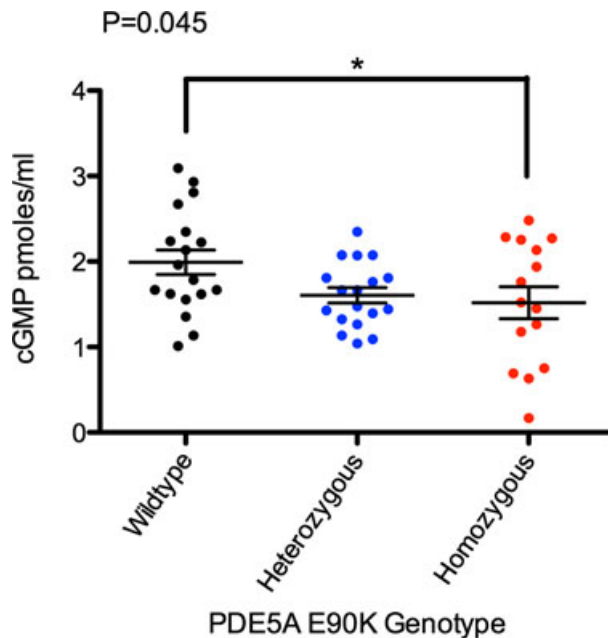


Fig 2. Basal cyclic guanosine monophosphate (cGMP) concentrations of healthy dogs are charted by genotype (wild type, heterozygous, homozygous). The trend of decreased cGMP concentration with each copy of the variant allele is observed and the overall *P*-value for the one-way ANOVA demonstrates significant difference. The asterisk denotes the statistical difference determined by Dunnett's test of multiple comparisons ($P < .05$) when comparing wild type with homozygous genotype groups.

($P < .05$; Fig 2). Heterozygous versus homozygous variants were not significantly different. Overall, dogs possessing the PDE5A:E90K polymorphism of either the heterozygous or homozygous genotype had lower basal cGMP concentrations in plasma.

Discussion

We hypothesized that functional polymorphisms in PDE5A exist that may alter circulating cGMP concentrations in healthy dogs and identified 1 such polymorphism, PDE5A:E90K, which supports this hypothesis. The PDE5A:E90K polymorphism is functionally predicted by evaluation of conservation (Table 2) and multiple software programs (Polyphen 2, Mutation Taster) to be damaging with consequences in amino acid structure, charge, acid-base status, potential splice site alterations, and interference with the functional elements of the protein. The change identified lies adjacent to the GAF1 functional element of the protein and is in the first few nucleotides of exon 2. Although the change does not lie within the splice site, the structural prediction software suggests the possibility that structural and acid-base change in this region could interfere with splicing at the RNA level. To investigate this polymorphism in healthy dogs, basal cGMP concentrations were measured and correlated with polymorphism genotype. The polymorphic genotype was quite common in the canine population with more than 75% of the population identified possessing the polymorphism in either the heterozygous or homozygous fashion. Dogs possessing the polymorphism had lower basal, circulating cGMP concentrations when compared with wild type dogs.

Although the hypothesis was supported by this study, it has several limitations. Predicting the relevance of polymorphisms on protein structure and function is difficult and imperfect. Although the models utilized are well established and the prediction was ranked highly, modeling programs do not replace the need for RNA and protein expression analysis, which was not performed as part of this study and would be necessary to confirm that the reported functional protein predictions are accurate. Additionally, because RNA and protein expression were not evaluated, the associated change in plasma cGMP concentration identified in this study could represent either normal protein concentrations with altered protein function, directly altered protein concentrations or some combination of these possibilities. Although a significant difference was identified in plasma-circulating cGMP concentrations, the tissue concentration of cGMP would better represent the vasodilatory action of cGMP. Rather than performing cell culture and intracellular cGMP measurement, we chose to assess plasma cGMP concentration as a baseline comparison of genotype groups. This decision is supported by evidence that in other species, plasma cGMP concentration is significantly correlated with aortic tissue concentrations of cGMP in and changes in cGMP after pharmacologic phosphodiesterase inhibition continue to correlate well.²³ Additionally, the study was performed in healthy dogs and no medical intervention with phosphodiesterase inhibiting drugs, additional diagnostic tests or investigation into disease states was performed.

Additionally, a series of 5 linked polymorphisms in the 3' untranslated regions were identified. Although these changes were not predicted by Mutation Taster or Polyphen to be damaging and were not found to impact basal cGMP concentrations, we cannot rule out their functional role in other physiologic processes or disease states. A polymorphism within a different functional unit of the human PDE5A gene has been linked to similar findings reported here with canine PDE5A:E90K and decreased circulating cGMP concentrations. This human polymorphism is further described to blunt the magnitude of vasodilatory response to inhaled nitric oxide.⁵ Additionally, the role of the human PDE5A polymorphism is thought possibly to represent a risk factor for development and progression of certain disease states such as IgA nephropathy, essential hypertension and erectile dysfunction.^{5–7} In the case of the reported PDE5A:E90K polymorphism, polymorphic dogs that possess lower basal cGMP concentrations may generate less of a response to drugs, such as sildenafil citrate, that typically increase cGMP to cause vasodilatation. In this scenario, knowing that a patient possesses the polymorphic genotype could prompt the use of a different vasodilatory agent or drug class.

It has been reported in multiple studies that not only is the magnitude of response to PDE5A inhibitors, such as sildenafil citrate, weak and often unsuccessful in the treatment of canine PH, but it also is apparently variable on an individual dog basis.^{15–17} With the exception of few pharmacogenomics discovery reports, the concept of individualized medicine is quite new for veterinary health care, but may elucidate the physiologic mechanism for these observations.^{2–4} Although this study did not set out to identify the impact of this polymorphism in a diseased population, it may serve as a pilot study to suggest that the impact of this polymorphism in various disease states such as PH or systemic hypertension is reasonable. The scope of interest may even extend beyond that of cardiorespiratory disease because recent investigations into PDE5A have described it as an oncogene and possible therapeutic target in Hodgkin lymphoma and hepatic neoplasia.^{24,25} The canine PDE5A gene possesses a least 1 amino acid-altering polymorphism (PDE5A:E90K) that appears to confer the functional consequence of decreased cGMP concentrations in healthy dogs and warrants further investigation.

Footnotes

- ^a Nanodrop 2000, Thermo Scientific, Wilmington, DE
^b Taq DNA Polymerase, Fermentas Life Sciences, Waltham, MA
^c Prism 377 Sequencer, ABI, Foster City, CA
^d SeqMan Pro, DNASTar, Madison, WI
^e cGMP EIA, Biomedical Technologies, Stoughton, MA
^f Prism 6.0, Graphpad Software Inc, La Jolla, CA

Acknowledgements

This study was not supported by any grants or scholarships.

Conflict of interest: Authors disclose no conflict of interest.

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